# The *Mycobacterium avium* subsp. *paratuberculosis* 35 kDa protein plays a role in invasion of bovine epithelial cells

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*Mycobacterium avium* subsp. *paratuberculosis* (*M. paratuberculosis*) enters intestinal epithelial cells of cattle and other ruminants via a mechanism that remains to be fully elucidated. This study showed that a gene encoding the *M. paratuberculosis* 35 kDa major membrane protein (MMP) is expressed at a higher level in low-oxygen and high-osmolarity conditions that are similar to the environment of the intestine. In addition, cattle with Johne's disease produced antibodies against MMP, suggesting that the protein is present during infection. The gene encoding MMP was cloned and expressed as a fusion protein with the maltose-binding protein (MBP–MMP) in *Escherichia coli*. Rabbit antisera were raised against a *M. paratuberculosis* whole-cell sonicate and MMP-specific antibodies were purified from these sera by affinity chromatography. MMP was localized to the surface of *M. paratuberculosis* by immunoelectron microscopy and by immunoblot analysis of fractionated protein lysates. Both anti-MMP antibodies and MBP–MMP protein inhibited *M. paratuberculosis* invasion of cultured Madin–Darby bovine kidney cells by 30 %. In similar invasion experiments with *M. paratuberculosis* incubated in low oxygen tension, these antibodies and protein decreased invasion by 60 %. Collectively, these data show that the 35 kDa MMP is a surface exposed protein that plays a role in invasion of epithelial cells. The authors suggest that the MMP is a virulence factor of *M. paratuberculosis* that may be important in the initiation of infection *in vivo*.

## INTRODUCTION

*Mycobacterium avium* subsp. *paratuberculosis* (*M. paratuberculosis*) is a facultative intracellular bacterium that infects both wild and domestic ruminant animals. Infections with this enteric pathogen are chronic, with decreased milk production, diarrhoea and weight loss occurring during advanced stages of the disease. Of primary concern to the dairy cattle and sheep industries is the economic significance of *M. paratuberculosis* infections of animals that result in Johne's disease. Based upon prevalence data and other information from animal producers, economic losses for the US dairy cattle industry alone exceed 200 million dollars annually (Ott *et al.*, 1999). The progression of Johne's disease begins with infection of young calves during suckling or feeding on pastures contaminated with *M. paratuberculosis*. A long subclinical phase ensues where the animals may shed bacilli in their faeces intermittently; however, no disease signs are present. This is followed by a clinical phase that results in diarrhoea and weight loss as the predominant signs.

Several pathogenic bacteria have exploited M cell function to gain entry into the subepithelium. These include *Salmonella enterica* subsp. *typhimurium* (Jepson & Clark, 2001; Jones *et al.*, 1994), *Yersinia pseudotuberculosis* (Clark *et al.*, 1998) and other enteric pathogens (Jepson & Clark, 1998). M cells are specialized epithelial cells involved in transport of antigens across mucosal epithelia to the underlying lymphoid tissues where protective immune responses are generated. Little is known about the manner in which *M. paratuberculosis* interacts with the intestinal mucosa in cattle and sheep. One study by Momotani *et al.* (1988) showed *M. paratuberculosis* entry in ligated ileal loops of calves and a second study with similar results was recently performed using ligated distal small intestine in sheep (Sigur-Dardottir *et al.*, 2001). Both of these studies demonstrated *M. paratuberculosis* entry through M cells. However, it is not certain whether all mycobacterial bacilli in

Abbreviations: MBP, maltose-binding protein; MDBK, Madin–Darby bovine kidney; MMP, major membrane protein.

a given infection use M cells as the sole route of entry. *Mycobacterium avium* subsp. *avium* (*M. avium*), an opportunistic pathogen with greater than 96 % nucleotide identity to *M. paratuberculosis*, invades the intestine of mice preferentially through enterocytes and not M cells (Sangari *et al.*, 2001). Specifically, Sangari *et al.* (2001) showed that the number of bacteria present in segmented intestinal tissue containing Peyer's patches was 100-fold less than that observed in non-Peyer's-patch regions.

A 35 kDa protein was originally identified in *Mycobacterium leprae* as an immunodominant antigen (Winter *et al.*, 1995). This antigen was termed the major membrane protein (MMP) because it was purified from a membrane fraction of *M. leprae*. Sera from patients with leprosy also recognized this MMP, further demonstrating its immunogenicity (Triccas *et al.*, 1996). This antigen was later found in *M. avium* (Banasure *et al.*, 2001; Triccas *et al.*, 1998) and *M. paratuberculosis* (Banasure *et al.*, 2001; Bannantine & Stabel, 2001). Recent studies with mice have suggested that DNA encoding the *M. leprae* MMP protects against leprosy infection (Martin *et al.*, 2001).

In the present study, we characterized the location and immunogenicity of the *M. paratuberculosis* MMP. In addition, a molecular cloning approach, combined with invasion assays, was used to determine if the MMP plays a role in invasion of bovine epithelial cells.

## METHODS

**Bacteria and culture conditions.** *M. paratuberculosis* type strain ATCC 19698 and bovine field isolate K-10 were grown in Middlebrook 7H9 broth or 7H10 agar supplemented with oleic acid/albumin/dextrose (glucose) complex (OADC; Becton Dickinson Microbiology), 0·05 % Tween 80 and ferric mycobactin J (2 mg l$^{-1}$). *E. coli* DH5α was cultured in LB broth and agar supplemented with 100 µg ampicillin ml$^{-1}$ where appropriate.

**Sera from Johne's disease cattle.** Sera from 13 naturally infected cattle in the clinical stage of Johne's disease and 4 healthy cattle were used in immunoblot assays for detection of antibodies that bind *M. paratuberculosis* MMP. All 13 infected cattle showed clinical signs of Johne's disease including diarrhoea and shedding of at least 30 *M. paratuberculosis* bacilli per g faeces. Affinity-purified MBP–MMP protein (see below) was used to test these sera for MMP-specific antibody. Cattle sera were diluted 1 : 500 and α-bovine immunoglobulin–HRP (Pierce Chemical Company) was the secondary antibody in these experiments.

**MMP expression in defined conditions.** To determine if MMP expression is increased when mycobacteria are exposed to conditions encountered in the intestinal lumen, transcript levels were measured by RT-PCR and translation was measured by relative protein abundance in defined conditions. An inoculum of 10$^7$ *M. paratuberculosis* was cultured in 7H9 broth and after the culture reached a turbidity equivalent to $5 \times 10^8$ organisms, it was split and placed at 37 °C in several defined environments: hyperosmolarity (complete 7H9 medium supplemented with 0·3 M glucose), anaerobiosis (anaerobic jar), the combination of both conditions, low pH (pH 5·0 was maintained by supplementing 7H9 broth with sulfonate buffers with appropriate p$K_a$ values), iso-osmolar and aerobic conditions as control for 24 h.

Total bacterial RNA was obtained as previously reported (Bermudez *et al.*, 1993). Contaminating DNA was removed from the mycobacterial RNA preparations using DNase digestions performed on RNeasy mini-columns (Qiagen) by adding 82 Kunitz units of enzyme (Qiagen) and incubating the columns at room temperature for 15 min. As a check to ensure all DNA was removed by this method, a control PCR was performed on the RNA preparation. No PCR product was obtained in these controls. Total RNA was initially quantified by $A_{260}$ and quality determined by $A_{260}/A_{280}$. Ratios $\geqslant 1·8$ were considered acceptable. RNA was then analysed by gel electrophoresis to confirm quality. RNA was submitted to reverse transcriptase treatment to obtain cDNA. Briefly, 6 µl RNA, 6 µl random hexamer (50 µg in 3 µl), 2 µl dNTP mix (10 mM) and distilled water were mixed and incubated at 65 °C for 5 min and subsequently placed on ice for 2 min (RT mix). Then to 18 µl of the RT mix, we added 5 µl 10 × RT buffer, 8 µl MgCl$_2$ (25 mM), DTT (0·1 M) and RNase (2 µl). The mix was incubated at 25 °C for 2 min and 2 µl SuperScript RTII (Invitrogen Life Technologies) was added. The sample was incubated for 10 min at 25 °C and then transferred to 42 °C for 50 min. The reaction was then placed on ice, centrifuged briefly and 2 µl RNase H was added followed by incubation at 37 °C for 20 min. The 16S RNA gene was used as the constitutively expressed control. To amplify the cDNA, we used specific primers for the MMP coding sequence and *M. paratuberculosis* genomic DNA. PCR amplification was carried out at 95 °C for 3 min (1 cycle), 95 °C for 3 s, 62 °C for 30 s, 72 °C for 2 min (35 cycles), and 72 °C for 10 min (1 cycle). Comparison of each amplified cDNA sample with control was carried out using 15 µl of equal amounts of cDNA.

An identical set of *M. paratuberculosis* cultures was processed as sonicated protein extracts in order to measure relative abundance of MMP in these defined conditions. Mycobacteria were harvested (8000 **g** for 30 min) and washed twice with cold phosphate-buffered saline. The pellet was resuspended in 0·01 of the culture volume and sonicated on ice with a probe sonicator (Tekmar sonic disruptor). Sonication consisted of three 10 min bursts at 18 W (highest setting) on ice with 10 min chilling periods in between. Debris was centrifuged (12 000 **g** for 15 min) and supernatants were combined and aliquoted at −20 °C. Protein concentration was determined using the Bio-Rad protein assay.

**Cloning and expression of the *M. paratuberculosis* MMP in *E. coli*.** A maltose-binding protein (MBP) fusion of MMP (MBP–MMP) was constructed using the pMAL-c2 vector (New England Biolabs). The reading frame was amplified using *Pwo* polymerase (Boehringer Mannheim) and *M. paratuberculosis* ATCC 19698 genomic DNA as the template. The upstream and downstream oligonucleotide primers for this amplification contain restriction sites for cloning (underlined) and are: 5′-GCGCAGGCCTGATCTGGCTGC-CAGGCAACTC-3′ and 5′-GCGCAAGCTTCACTTGTACTCATGG-AACTG-3′. This primer combination produces the MMP coding sequence minus the initial 12 codons (see GenBank accession number AF232751). The vector was digested sequentially with *Xmn*I and *Hin*dIII, and the amplification product was digested with *Stu*I and *Hin*dIII. Ligation of these products resulted in an in-frame fusion between the vector-encoded *malE* gene and a majority of the *mmp* gene. The resulting recombinant was designated pMAL-mmp. Following ligation, pMAL-mmp was transformed into *E. coli* DH5α. The insert from the construct was confirmed by DNA sequencing. The resulting fusion protein was overexpressed and purified by maltose affinity chromatography using an amylose resin from New England Biolabs. The purified MBP–MMP fusion protein was used as antigen in subsequent experiments. An *E. coli* clone (275-1), producing MMP without the MBP affinity tag, was identified previously from a *M. paratuberculosis* lambda phage expression library (Bannantine & Stabel, 2001).

**Antibody production and purification.** Two New Zealand White rabbits were immunized with a sonicated protein lysate of *M. paratuberculosis* using an immunization regimen described previously (Stabel *et al.*, 1996). TiterMax (CytRx Corp.) served as the adjuvant in these experiments. MMP monospecific antibodies were affinity purified from rabbit sera raised against *M. paratuberculosis* using AminoLink columns (Pierce Chemical Company). Briefly, the MBP–MMP protein was coupled by reductive amination to a 4% agarose support column (Pierce Chemical Company). Antisera from the immunized rabbits (1–2 ml) were passed over the column followed by three washes and elution according to the instructions of the manufacturer. Eluted fractions were evaluated by spectrophotometry and immunoblot analysis (Bannantine & Stabel, 2001). Fractions with the highest $A_{280}$ and the strongest reactivity by immunoblotting were neutralized by addition of 1 M Tris/HCl (pH 9·5) buffer and then dialysed at 4 °C in phosphate-buffered saline. For immuno-electron microscopy, colloidal gold-conjugated goat anti-rabbit IgG was purchased from Ted Pella, Inc.

For production of monoclonal antibody, 6–7-week-old BALB/c mice were immunized with MBP (30 μg in 150 μl for each mouse). Splenic lymphocytes from immunized mice were harvested and fused to SP2/0 myeloma cells (Harlow & Lane, 1988). Because of solubility problems associated with the MBP, each well was screened by preparative immunoblotting using MBP as antigen. Positive cell lines were cloned and monoclonal antibodies harvested in hybridoma culture supernatants. Monoclonal antibody (13A4) against a *M. paratuberculosis* 60 kDa protein was developed using similar methods. Appropriate anti-mouse IgG secondary antibodies were purchased from Pierce Chemical Company.

**Electrophoresis and immunoblotting.** *E. coli* lysates expressing MMP or MBP–MMP were prepared as previously described (Rockey & Rosquist, 1994). PAGE was performed using 12% (w/v) polyacrylamide gels. Electrophoretic transfer of proteins onto nitrocellulose (Schleicher and Schuell) was accomplished with the Bio-Rad Trans Blot Cell (Bio-Rad) in sodium phosphate buffer (25 mM, pH 7·8) at 0·9 A for 90 min. After transfer, filters were blocked with phosphate-buffered saline (PBS; 150 mM NaCl, 10 mM sodium phosphate, pH 7·4) plus 2% bovine serum albumin (BSA) and 0·1% Tween 20, referred to hereafter as PBS-BSA. Monospecific MMP antibodies were diluted 1:1500 in PBS-BSA and incubated on the blot at room temperature for 2 h. After three washes in PBS plus 0·1% Tween 20, blots were incubated for 1·5 h in protein A–peroxidase (Pierce Chemical Company) diluted 1:20 000 in PBS-BSA. The blots were again washed three times as described above and developed for chemiluminesence using Supersignal detection reagents (Pierce Chemical Company).

**Subcellular fractionation of *M. paratuberculosis*.** In order to test the location of MMP within *M. paratuberculosis*, cell fractions were obtained from sonicated preparations and analysed by immunoblotting using affinity-purified MMP antibodies. The pellet from a high-speed centrifugation (90 000 *g* for 60 min at 4 °C) was collected and represented the membrane/cell wall fraction. The supernatant, saved following the high-speed centrifugation and representing the cytosolic proteins, was concentrated in a Savant Speedvac. Both fractions were resuspended in SDS-PAGE loading buffer and an amount of protein equivalent to 450 μl of culture was subjected to SDS-PAGE and immunoblotting. The Middlebrook 7H9 culture supernatant was similarly concentrated and processed to test for secretion of MMP.

**Immunoelectron microscopy.** All fixation and staining procedures were conducted at room temperature. *M. paratuberculosis* ATCC 19698 cells were cultured for 4 weeks in Middlebrook 7H9 medium containing OADC and mycobactin J. Cells were fixed for 2–4 h in 2·5% glutaraldehyde in 0·1 M cacodylate buffer, pH 7·4.

Fixed cells were washed in the same buffer three times and postfixed in 1% $OsO_4$ in 0·1 M cacodylate buffer, pH 7·4, for 2 h. After washing in the same buffer, cells were incubated with 30% ethanol for 10 min. The cells were further dehydrated with a graded series of ethanol and embedded in epoxy resin (Embed 812). Ultrathin sections for immunoelectron microscopy were washed in buffer for 15 min three times and etched with saturated sodium metaperiodate for 15 min. The sections were then blocked with 5% BSA for 30 min at room temperature, followed by treatment with purified MMP-specific antibodies (diluted 1:100) in the blocking solution for 2 h at room temperature. The sections were washed in Tris buffer containing 0·1% Tween 20 and 0·1% BSA four times for 10 min each and then incubated with goat anti-rabbit IgG conjugated to colloidal gold (10 nm diameter) in Tris buffer for 2 h. Immunolabelled sections were washed in Tris buffer four times and fixed with 1% glutaraldehyde in Tris buffer for 10 min. All ultrathin sections were double stained with uranyl acetate and Reynolds lead citrate and then observed under a Philips 410 microscope.

**Invasion assay.** To test bovine epithelial cell invasion *in vitro*, Madin–Darby bovine kidney (MDBK) cells were used. MDBK cells were cultured to 80% confluence in RPMI 1640 plus Dulbecco's Minimal Essential Medium supplemented with 10% heat-inactivated fetal bovine serum. MDBK cells in culture for approximately 4 days were incubated with specific antibody or purified protein for 30 min prior to addition of $10^6$ c.f.u. of *M. paratuberculosis* in Hanks' Balanced Salts Solution (HBSS; bacteria:cell ratio 1:1). To avoid clumping, the bacterial suspension was passed through a 16 gauge needle 10 times and then placed in a 15 ml tube and allowed to rest on the bench for 10 min. Only the top half of the suspension was used in the assays. An aliquot of the bacterial suspension was stained using the LIVE and DEAD assay (Molecular Probes) and only disperse inocula with more than 85% of viable bacteria were used in the assays. After 2 h at 37 °C in 5% $CO_2$, monolayers were washed twice with HBSS to remove extracellular bacteria and then treated with amikacin (200 μg ml$^{-1}$) for 2 h at 37 °C. Amikacin at this concentration either kills or detaches the extracellular bacteria without any effect on the viability of the intracellular bacteria (Bermudez *et al.*, 1997). Supernatant was removed and monolayers lysed with 0·1% Triton X-100 for 15 min. Lysates were then added to 0·025% SDS for 10 min, serially diluted and plated onto Middlebrook 7H10 agar slants containing mycobactin J.

**Statistical analysis.** The results of experimental groups obtained from at least three invasion assays were compared with the controls at the same time point. A statistical analysis of these comparisons was done by using the Student's *t* test. $P < 0·05$ was considered significant.

# RESULTS

## MMP transcription is increased when mycobacteria are exposed to specific environmental conditions

The gene encoding MMP may alter expression levels in the environment encountered in the intestinal lumen. To address this question, expression of the MMP coding sequence was examined in *M. paratuberculosis* exposed to low oxygen tension, high osmolarity, acidic pH or the combination of low oxygen and increased osmolarity. RT-PCR amplification results shown in Fig. 1 demonstrate that under high osmolarity and low oxygen tension MMP transcription is increased when compared to acidic pH or standard aerobic growth conditions. These results are
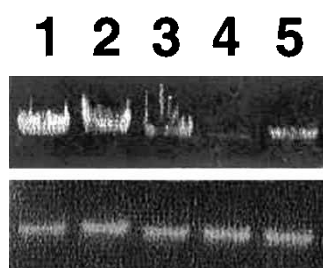
**Fig. 1.** RT-PCR analysis of *mmp* under specific environmental conditions. Bacteria were exposed to low oxygen tension (lane 1), increased osmolarity (lane 2), both low oxygen and increased osmolarity (lane 3), pH 5 (lane 4), and standard iso-osmolar and aerobic culture conditions (lane 5) for 24 h. Then RNA was extracted, reverse-transcribed and the cDNA was used as template for amplification of the gene encoding the 35 kDa protein (MMP) (upper panel) and 16S RNA (lower panel).

similar to those obtained previously with a homologous gene in *M. avium* (L. E. Bermudez & J. P. Bannantine, unpublished results).

## Characterization of recombinant MMP

In order to obtain purified quantities of protein, *M. paratuberculosis* MMP was produced in *E. coli* as a fusion with MBP and affinity-purified from recombinant *E. coli* lysates. Because MBP was not efficiently cleaved from the fusion protein after digestion with factor Xa protease

(data not shown), all experiments were performed using the intact fusion protein. Antibodies to MMP were affinity-purified from rabbit sera as described in Methods.

MBP–MMP was analysed by SDS-PAGE and immunoblotting to assess purity and further characterize the fusion protein (Fig. 2A–C). Lane 2 of Fig. 2(A) shows a single band at 75 kDa indicating the purity of the fusion protein. The calculated size of MMP alone is 34 kDa and that of MBP is 42 kDa. MBP–LacZ α peptide was purified and used as a control in these experiments (lane 1). This control protein migrated at 50 kDa (42 kDa MBP plus the 8 kDa LacZ α peptide). An *E. coli* clone (275-1) expressing MMP without the MBP affinity tag was previously identified from a *M. paratuberculosis* phage expression library (Bannantine & Stabel, 2001). This recombinant *E. coli* lysate was loaded in lane 3 of Fig. 2(A–C). One immunoblot was probed with a monoclonal antibody that detects only the MBP tag of each fusion protein in lanes 1 and 2 (Fig. 2B). A second identical immunoblot (Fig. 2C), probed with affinity purified MMP-specific antibodies, detects only the MMP portion of the MBP–MMP fusion (lane 2) as well as MMP in the recombinant *E. coli* 275-1 lysate (lane 3).

We next determined if MMP-specific antibodies would identify the protein in *M. paratuberculosis* protein lysates. Immunoblot analysis shows that these antibodies identified a 35 kDa protein in both the type strain of *M. paratuberculosis* (ATCC 19698) and K-10, a bovine isolate selected for the genome sequence project (J. P. Bannantine, V. Kapur, L.-L. Li & Q. Zhang, unpublished; Fig. 2D). A
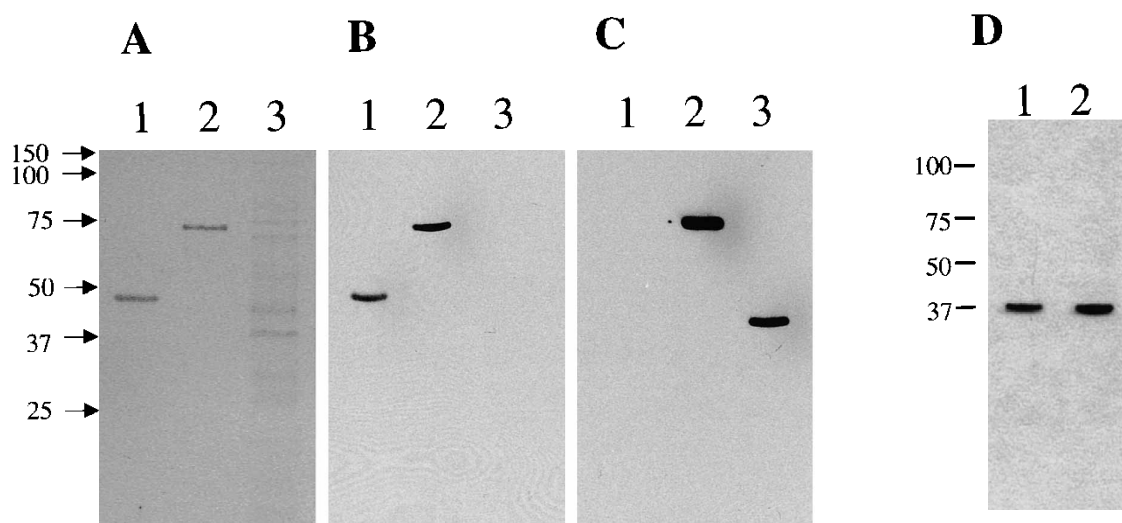


**Fig. 2.** SDS-PAGE and immunoblot analysis of the MMP expressed in *E. coli*. (A–C) SDS-PAGE gel (A) and two corresponding immunoblots (B, C) of purified MBP (lane 1), purified MBP–MMP fusion protein (lane 2), and lysate of *E. coli* 275-1 expressing MMP (lane 3). Identical amounts of protein are present in all three images. Immunoblot (B) was probed with a monoclonal antibody against MBP and immunoblot (C) was probed with affinity-purified MMP-specific antibodies. (D) Immunoblot of *M. paratuberculosis* sonicated protein lysates probed with MMP-specific antibodies. Lanes: 1, *M. paratuberculosis* ATCC 19698; 2, *M. paratuberculosis* K-10. Size standards (kDa) are indicated in the left margin of (A) and (D).

monoclonal antibody to MBP did not detect any *M. paratuberculosis* proteins from either strain, showing that MBP is not produced in these bacilli (data not shown). These immunoblot data show that MMP-specific antibodies do not cross-react with other *M. paratuberculosis* proteins.

## MMP protein is present in higher relative abundance in selected conditions

With specific antibodies to MMP obtained, we again addressed the question of MMP expression in low-oxygen and hyperosmolarity conditions. *M. paratuberculosis* was cultured in defined conditions for 24 h as described in Methods. Immunoblot analyses using the specific MMP antibodies were conducted in duplicate to assay for MMP abundance in *M. paratuberculosis* protein lysates produced from each condition. The results of these experiments show that a higher relative abundance of MMP is produced by *M. paratuberculosis* in low oxygen as compared to bacilli grown in aerobic conditions (Fig. 3). The relative abundance of MMP appears to decrease slightly in hyperosmolarity conditions but is still higher than that observed in aerobic cultures (Fig. 3). A 60 kDa protein served as an internal control to normalize MMP abundance because its expression is unaffected by oxygen and osmolarity (data not shown). Collectively, these experiments show that both transcription and translation of MMP is increased when exposed to these conditions.
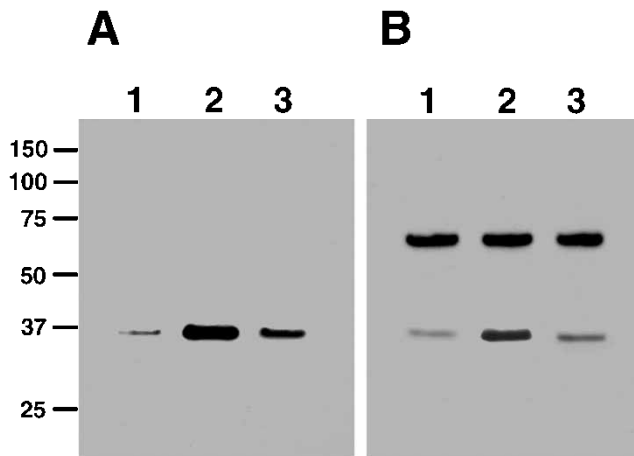


**Fig. 3.** Effect of oxygen and osmolarity on abundance of MMP in *M. paratuberculosis*. Protein lysates were produced from *M. paratuberculosis* cultured in aerobic conditions (lane 1) or exposed to low oxygen (lane 2), or hyperosmolarity (lane 3) for 24 h. An immunoblot assay was performed in duplicate where one immunoblot was exposed to purified MMP antibodies (A) and a second immunoblot was exposed to both MMP antibodies and a monoclonal antibody (13A4) to a 60 kDa *M. paratuberculosis* protein (B). The 60 kDa *M. paratuberculosis* protein was used to normalize relative abundance of MMP. Protein size standards are shown in kDa in the left margin.

## MMP is a surface protein of *M. paratuberculosis*

Although no known N-terminal signal peptide was detected, a Kyte–Doolittle hydropathy plot analysis (Kyte & Doolittle, 1982) of MMP shows a 30 amino acid hydrophobic domain near the C-terminal end suggestive of a membrane protein. To directly determine the subcellular location of MMP, we performed immunoelectron microscopy analysis on *M. paratuberculosis* using MMP-specific antibodies. Transmission electron microscopy revealed a majority of the colloidal-gold label at the periphery of the bacilli, which indicates that MMP is a surface-exposed protein (Fig. 4A). Intense labelling was observed in thin sections where a portion of the cell wall had been sheared from the rest of the bacilli, making surface proteins more readily available for antibody binding (Fig. 4B). In contrast, the MBP–MMP fusion protein was not localized to the surface of recombinant *E. coli* (data not shown). Therefore, invasion studies were not performed using these recombinant *E. coli*. Similar to the immunoblot results, MBP monoclonal antibodies did not label *M. paratuberculosis* bacilli in immunoelectron microscopy experiments (data not shown). A second set of experiments was performed to independently confirm the subcellular location of MMP. Sonicated extracts of *M. paratuberculosis* were separated into cytoplasmic and membrane/cell wall fractions as described in Methods. These fractions were then analysed for the presence of MMP by immunoblot analysis (Fig. 4C). MMP was associated exclusively with the membrane/cell wall fraction. Collectively, these data show that MMP is surface exposed in *M. paratuberculosis*.

## Immunological reactivity of MMP

Because MMP is a surface protein in mycobacteria, it was of interest to determine if this protein elicits antibody production in infected cattle. An analysis of the host antibody response to MMP was performed using samples from healthy control cattle and animals naturally infected with *M. paratuberculosis*. Immunoblot analysis with purified fusion protein was used to determine if sera from infected cattle recognized MMP. Sera from 8 of 13 infected cattle in the clinical stage of Johne's disease recognized only the MBP–MMP fusion protein but not MBP alone (Fig. 5). Therefore, those cows possess antibody against MMP. Sera from two infected cows did not react with either MMP or MBP, while sera from three infected cows reacted with MBP (Fig. 5A). Detection of only the MMP portion of the fusion protein with serum from one clinical cow is shown in a representative SDS-PAGE and immunoblot experiment in Fig. 5(B). Reactivity to MMP and MBP was not detected in healthy control cows. These data suggest that MMP is immunogenic in the context of *M. paratuberculosis* infection of cattle.

## MMP plays a role in invasion of bovine epithelial cells

Because *M. paratuberculosis* MMP shows increased expression in conditions that may mimic the intestine, and MMP
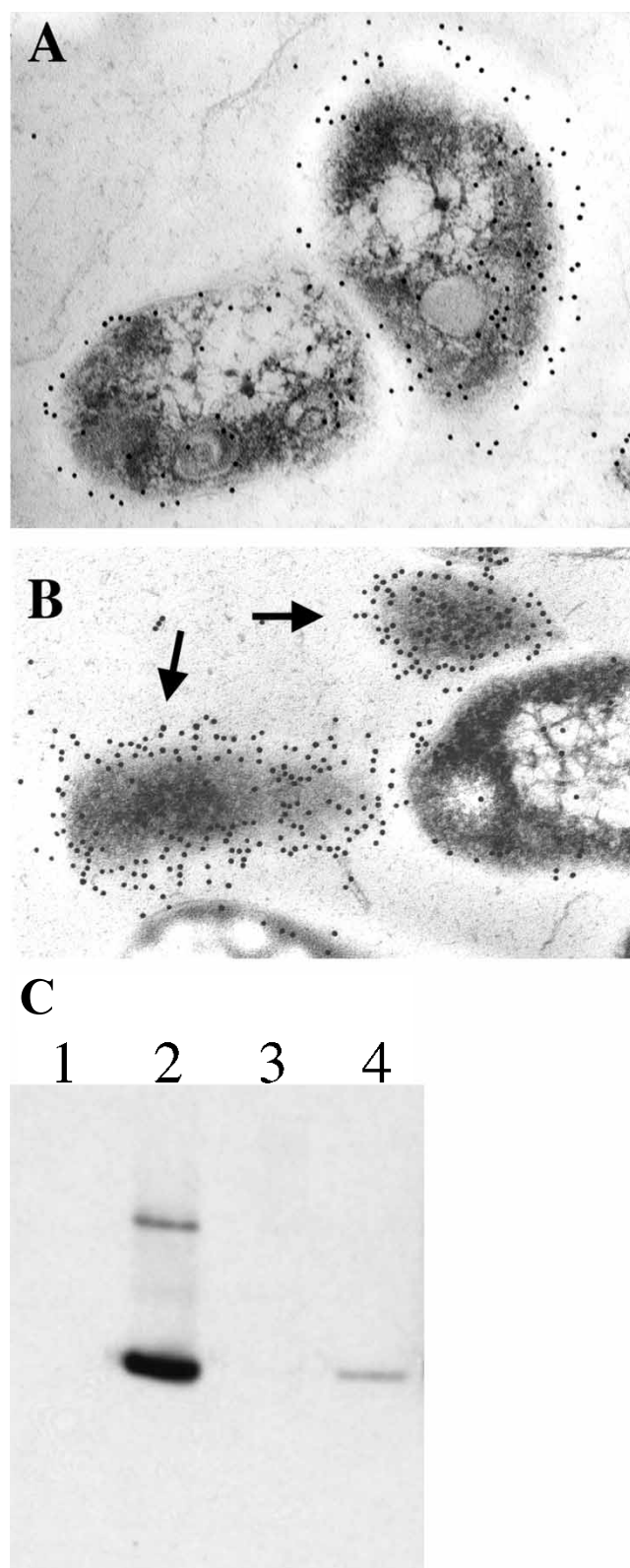
**Fig. 4.** Immunoelectron microscopy (A, B) and immunoblot analysis of *M. paratuberculosis* fractions (C) with MMP-specific antibodies shows that MMP is present on the surface of *M. paratuberculosis*. Shown in (A) are thin sections of two *M. paratuberculosis* bacilli labelled with MMP-specific antibodies. The location of antibody binding is shown using 10 nm colloidal gold particles. The majority of gold particles appear at the periphery of the bacilli. The intense labelling of sheared *M. paratuberculosis* bacilli is identified by arrows in (B) and indicates strong antibody binding in thin sections where only the cell wall is exposed. Magnification 94 000 ×. (C) Immunoblot of *M. paratuberculosis* extracts fractionated into cytosol and membrane/cell wall fractions. The immunoblot was exposed to MMP-specific antibodies. Lanes: 1, culture medium supernatant; 2, membrane/cell wall fraction; 3, cytosolic fraction; 4, *M. paratuberculosis* whole-cell lysate.

invasion of cultured MDBK cells in a dose-dependent manner. As shown in Table 1, the ability of *M. paratuberculosis* to invade MDBK cells is inhibited as the concentration of MBP–MMP is increased. Similarly, the percentage invasion decreases as the concentration of MMP-specific antibody increases. In contrast, approximately 7 % of the inoculum invaded MDBK cells in the no-treatment, MBP and nonspecific rabbit antiserum controls. We next evaluated the ability of *M. paratuberculosis* invasion to be abrogated by MBP–MMP and MMP-specific antibodies when the bacilli had been incubated in low oxygen tension for 4 h prior to invasion. The data show that this competitive effect was even more pronounced when *M. paratuberculosis* was held in low oxygen tension (Table 2). Therefore, when oxygen is present, *M. paratuberculosis* invasion was inhibited by 30 %, whereas in low-oxygen conditions, invasion was inhibited by 60 %. These data show that MMP plays a role in invasion of bovine epithelial cells and that this role is emphasized in oxygen conditions that are similar to those within the intestine.

## DISCUSSION

Many enteric pathogens are capable of directing their uptake into host epithelial cells lining the intestine. Previous studies have shown that *M. avium*, a close relative of *M. paratuberculosis*, is well adapted to the environmental conditions encountered within the intestine and that both hyperosmolarity and anaerobiosis significantly increase the efficiency of *M. avium* entry into HT-29 cells (Bermudez *et al.*, 1997). However, no specific genes were identified that respond to these environmental stimuli and play a role in invasion. In this report, we describe the heterologous expression and characterization of a 35 kDa *M. paratuberculosis* surface protein that is regulated in low-oxygen and hyperosmolarity conditions. In addition, we show evidence for its role in the invasion of MDBK cells, a role that is increased in low oxygen.

*M. paratuberculosis* invades the bovine intestinal mucosa as a first step in the pathogenesis of Johne's disease. The

is surface located, we hypothesized that it may play a role in invasion of bovine intestinal epithelial cells. To test this hypothesis, we evaluated the ability of purified proteins and MMP-specific antibodies to block *M. paratuberculosis*
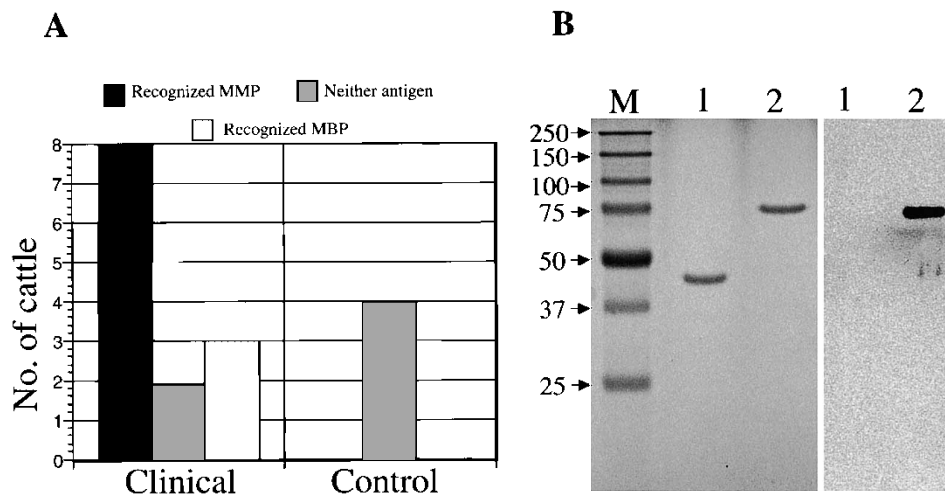
**Fig. 5.** MMP is detected by sera from cattle with Johne's disease. (A) Graphical representation of immunoblot data showing the number of infected and healthy control cattle that recognized MBP–MMP, MBP or neither antigen. Sera from 13 clinical cows and 4 control cows were analysed. (B) A representative SDS-PAGE gel (left image) and immunoblot (right image). The Coomassie-stained SDS-PAGE gel shows the position and relative amounts of MBP (lane 1) and MBP–MMP fusion protein (lane 2). The right panel is a corresponding immunoblot probed with serum from clinical cow no. 47. This serum detects the MBP–MMP fusion but not the MBP affinity tag alone. Protein size markers are in lane M and their kDa sizes are indicated in the left margin.

interaction between *M. paratuberculosis* and the bovine intestinal mucosa has been described to occur through M cells (Momotani *et al.*, 1988), although it is plausible that the bacterium crosses the intestinal wall by invading intestinal epithelial cells as well. *M. paratuberculosis* surface proteins probably play the dominant role in the initial interactions with bovine or ovine intestinal cells. Until very recently, no *M. paratuberculosis* surface proteins had been described. MMP is currently one of only two surface proteins identified

for *M. paratuberculosis* (Secott *et al.*, 2002). We have demonstrated that MMP is surface located using two independent methods. Despite the location of this protein, no predicted signal peptide was observed using the method of Krogh *et al.* (2001) and SignalP analysis (http://www.cbs.dtu.dk/services/SignalP/). This situation is not unprecedented, as other exported mycobacterial proteins do not posses a classical signal sequence (Pethe *et al.*, 2000; Thole *et al.*, 1995).

The majority of intestinal pathogens are able to regulate the expression of virulence factors once in the intestinal

**Table 1.** Blocking of *M. paratuberculosis* invasion of MDBK cells by specific antibodies or purified protein

| Treatment* | Concn | Invasion after 2 h (% of inoculum)† |
|---|---|---|
| None | | $7\cdot3 \pm 0\cdot2$ |
| MBP | $10\ \mu g\ ml^{-1}$ | $7\cdot4 \pm 0\cdot3$ |
| MBP–MMP | $0\cdot1\ \mu g\ ml^{-1}$ | $6\cdot2 \pm 0\cdot2$ |
| | $1\cdot0\ \mu g\ ml^{-1}$ | $5\cdot6 \pm 0\cdot2‡$ |
| | $10\ \mu g\ ml^{-1}$ | $5\cdot1 \pm 0\cdot4‡$ |
| MMP-specific antibodies | $1:40$ | $5\cdot7 \pm 0\cdot2‡$ |
| | $1:80$ | $5\cdot9 \pm 0\cdot1‡$ |
| | $1:160$ | $6\cdot5 \pm 0\cdot3$ |
| | $1:320$ | $6\cdot9 \pm 0\cdot2$ |
| Rabbit serum§ | $1:40$ | $7\cdot6 \pm 0\cdot4$ |

*Purified protein or antibodies were added 30 min prior to bacteria.
†Data are reported as the mean $\pm$ standard deviation of four experiments.
‡$P<0\cdot05$ compared with no treatment and MBP control.
§Serum obtained from a non-immunized rabbit.

**Table 2.** Blocking of *M. paratuberculosis* invasion of MDBK cells in low oxygen tension

| Treatment* | Concn | Invasion after 2 h (% of inoculum)† |
|---|---|---|
| None | | $10\cdot4 \pm 0\cdot3$ |
| MBP | $10\ \mu g\ ml^{-1}$ | $11\cdot4 \pm 0\cdot2$ |
| MBP–MMP | $10\ \mu g\ ml^{-1}$ | $4\cdot2 \pm 0\cdot3‡$ |
| MMP-specific antibodies | $1:40$ | $4\cdot5 \pm 0\cdot4‡$ |
| | $1:80$ | $5\cdot6 \pm 0\cdot2‡$ |
| | $1:160$ | $7\cdot6 \pm 0\cdot4$ |
| Rabbit serum§ | $1:40$ | $10\cdot1 \pm 0\cdot4$ |

*Purified protein or antibodies were added 30 min prior to bacteria.
†Data are reported as the mean $\pm$ standard deviation of three experiments.
‡$P<0\cdot05$ compared to no treatment and MBP control.
§Non-immunized rabbit serum.

environment. For example, *Salmonella* HilA, a global regulator of invasion of the intestinal mucosa, and *Yersinia ail* are both regulated in conditions of oxygen tension (Lee *et al.*, 1992; Pederson & Pierson, 1995). Similarly, osmolarity has been shown to be an environmental signal controlling the expression of genes associated with virulence in several other pathogens, among them the *toxR* gene of *Vibrio cholerae* (Miller & Mekalanos, 1988) and *ompR* genes of *Salmonella* and *Shigella* species (Bernardini *et al.*, 1990; Chatfield *et al.*, 1991). *M. avium*, a pathogen that is mainly acquired through the gastrointestinal tract (Bermudez *et al.*, 1997), has been demonstrated to have its uptake by human intestinal epithelial cells increased by several-fold when it is pre-incubated in low oxygen tension or hyperosmolarity, two conditions encountered in the intestinal tract.

In *M. paratuberculosis*, RNA transcription and protein expression data show that MMP expression is increased in both low-oxygen and hyperosmolarity conditions. It is plausible to assume that *M. paratuberculosis* is subject to the influence of the intestinal environment and therefore regulates proteins associated with the interaction of the mucosa accordingly. The present study also shows that the role of MMP in invasion is increased when *M. paratuberculosis* is exposed to low-oxygen conditions. A host immune response is produced against MMP, giving further evidence of its *in vivo* expression. We show that MMP elicits antibody production in infected cattle, which is consistent with observations that show MMP is recognized in sera from leprosy patients (Triccas *et al.*, 1996).

Entry into the mucosal surface is a complex phenomenon that in many pathogens involves the participation of a number of virulence genes. We used both the purified MMP protein and antibody to show a specific but small diminution in invasion. It is clear from these data that MMP is a player probably among several moieties and it is not surprising that the differences in invasion are not greater, though they are statistically significant. Regarding the level of invasion of *M. paratuberculosis*, it is similar to that observed with other mycobacteria (Bermudez *et al.*, 1997; Sangari *et al.*, 2001).

MMP is not the first mycobacterial invasion protein described. A recent report by Secott *et al.* (2002) showed that decreased expression of the fibronectin attachment protein, FAP-P, reduced attachment and ingestion of *M. paratuberculosis* in Caco-2 cells. In addition, a 21 kDa *M. leprae* protein has been shown to mediate invasion of Schwann cells of the peripheral nerves (Shimoji *et al.*, 1999). In that study, the 21 kDa protein was coated onto polystyrene beads and shown to invade in a concentration-dependent manner after 12 h using fluorescence and electron microscopy. Another *M. leprae* protein, which binds fibronectin, has been found to play a role in invasion using a strategy similar to that described here (Schorey *et al.*, 1995). *M. tuberculosis* also produces a protein shown to promote entry into mammalian cells (Arruda *et al.*, 1993; Flesselles *et al.*, 1999). This protein, termed Mce, conferred invasiveness upon a non-pathogenic *E. coli* strain (Arruda *et al.*, 1993). The *M. paratuberculosis* 35 kDa protein is present in *M. leprae* (Triccas *et al.*, 1996; Winter *et al.*, 1995) and *M. avium* (Triccas *et al.*, 1998), but is absent in *M. tuberculosis* and *M. bovis* (Banasure *et al.*, 2001; Triccas *et al.*, 1998). *M. avium* has been shown previously to invade intestinal epithelial cells (Sangari *et al.*, 2001) and, although not demonstrated by experiments reported in this study, MMP may play a role in these interactions as well (Miltner *et al.*, 2001).

In summary, we have identified a novel *M. paratuberculosis* protein associated with the invasion of epithelial cells. Future studies involving monoclonal antibody production and protein interaction studies between MMP and MDBK cells are under way.

## ACKNOWLEDGEMENTS

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